

Generation of Nanobodies against SlyD and development of tools to eliminate this bacterial contaminant from recombinant proteins



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ABSTRACT

The gene for a protein domain, derived from a tumor marker, fused to His tag codons and under control of a T7 promotor was expressed in *E. coli* strain BL21 (DE3). The recombinant protein was purified from cell lysates through immobilized metal affinity chromatography and size-exclusion chromatography. A contaminating bacterial protein was consistently co-purified, even using stringent washing solutions containing 50 or 100 mM imidazole. Immunization of a dromedary with this contaminated protein preparation, and the subsequent generation and panning of the immune Nanobody library yielded several Nanobodies of which 2/3 were directed against the bacterial contaminant, reflecting the immunodominance of this protein to steer the dromedary immune response. Affinity adsorption of this contaminant using one of our specific Nanobodies followed by mass spectrometry identified the bacterial contaminant as FKBP-type peptidyl-prolyl *cis-trans* isomerase (SlyD) from *E. coli*. This SlyD protein contains in its C-terminal region 14 histidines in a stretch of 31 amino acids, which explains its co-purification on Ni-NTA resin. This protein is most likely present to varying extents in all recombinant protein preparations after immobilized metal affinity chromatography. Using our SlyD-specific Nb 5 we generated an immune-complex that could be removed either by immunocapturing or by size exclusion chromatography. Both methods allow us to prepare a recombinant protein sample where the SlyD contaminant was quantitatively eliminated.

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1. Introduction

Escherichia coli bacteria are widely used as expression host for recombinant proteins [1,2] due to its low cost of fermentation and above all a profound understanding of its genetics, the availability of efficient transformation tools, the possibility to regulate expression under tight control of strong promoters, etc. Subsequent to the protein expression in *E. coli*, multiple strategies have been developed to purify the recombinant protein of interest. The introduction of immobilized metal affinity chromatography (IMAC) has revolutionized the protein purification process. The presence of a small peptide tag (e.g., hexa histidine) at the C- or N-terminal end

of the recombinant protein allows the specific binding to immobilized bivalent metal ions [3,4]. After washing off the non-bound molecules in physiological solutions, the adsorbed recombinant protein is eluted in solutions containing imidazole or by lowering the pH [5–7]. Perfect stability of metal ion-loaded resin and a high protein loading capacity support the widespread application of IMAC to purify recombinant proteins in both low and high throughput settings at an affordable cost [8,9]. Many different metal-chelator systems have been developed for IMAC, including the most commonly used Ni²⁺ bound to tetradentate ligand NTA (Ni-NTA) [10,11] and Co²⁺ bound to tetradentate ligand CM-Asp (TALON™) [12].

However, the occurrence of endogenous bacterial proteins with an affinity for divalent metal ions frequently results in their co-purification with the recombinant protein on Ni-NTA [13] and TALON columns [14]. Even after a subsequent size exclusion

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chromatography (SEC) [15], these proteins might still contaminate the recombinant protein sample. Most of these metal-binding proteins are present in *E. coli* strains of different genetic backgrounds, such as BL21, BL21 (DE3), BL21 (DE3) pLysS and Shuffle T7 cells. One of these proteins is FKBP-type peptidyl-prolyl *cis-trans* isomerase (SlyD) [16–18]. Histidine residues represent close to 10% of the amino acids within SlyD, which explains its affinity for Ni-NTA resins [19,20]. In cases where the recombinant protein has a MW close to that of SlyD, a SEC will fail to separate these molecules. Evidently, the presence of such a contaminating protein will interfere with the subsequent in-depth analysis of the target recombinant protein. The introduction of additional washing steps including organic solvents (e.g. isopropanol) to remove these contaminants has been proposed [21]. However, they often lead to destabilization and eventual denaturation of the recombinant protein and concomitantly to lower yields. Therefore the best approach would be to develop a strategy to capture the contaminant proteins from a recombinant protein preparation, and antibodies might be most suitable candidates for this purpose.

The heavy chain-only antibodies (HCAbs), naturally occurring in camelids [22] are a good starting point to develop such an affinity adsorbent. The antigen-binding fragment of HCAs is comprised in the variable domain of the heavy chain (VHH), also known as Nanobody (Nb). It is the smallest, intact, functional antigen-binding fragment and has been used widely in different applications [23–26]. The Nbs are generally well expressed at low cost in microbial systems, and normally exhibit high affinity and specificity for their cognate antigen. Nbs are also highly stable and soluble in aqueous solutions even at elevated concentration [27,28] and often recognize epitopes that are less antigenic for classical antibodies comprising heavy and light chains. A steadily growing number of groups are generating and using Nbs in the area of cancer diagnostic or in cancer immunotherapy [23]. In the current study, an immune Nb library against the recombinant PasB domain (rPasB, ~15 kDa) [29,30] was constructed in our in-house phage display vector. After phage display and panning against rPasB, we retrieved several Nbs that recognized a contaminating protein of ~25 kDa in western blot. This contaminant was identified by LC-MS/MS as *E. coli* SlyD. Our Nbs are thermostable proteins that recognize specifically the SlyD protein, in several *E. coli* lysates with single digit nM affinity. With one of these Nbs, we developed two approaches to quantitatively remove SlyD from contaminated recombinant protein preparations. To treat larger batches of recombinant protein extracts, an affinity adsorbent could be developed.

2. Material and methods

2.1. Bacterial strains and culture conditions

E. coli strain BL21 (DE3) was used as host to produce the recombinant Pas B domain (rPasB) from the pET21b plasmid, containing a T7 promoter. *E. coli* TG1 cells were used to construct the VHH library, for phage display and to screen for presence of antigen-specific Nbs by ELISA. *E. coli* WK6 cells were used to produce Nbs with a hemagglutinin tag (HA-tag) and a His-tag at their C-terminal end. The same WK6 cells were used to host the BirA producing plasmid together with the pBAD17 plasmid for *in vivo* expression of biotinylated Nb (Bio-Nb) under control of a lac promoter. Luria Bertani broth (LB) medium was used to grow TG1 cells at 37 °C, while 2×TY medium was preferred to grow TG1 cells during phage display experiments. Terrific Broth (TB) was used for the expression and production of Nbs and rPasB. All the media were supplemented with ampicillin (100 µg/ml) to maintain the pressure for the hosted plasmids.

2.2. Preparation of recombinant antigen

The PasB domain comprises amino acids 238–343 from the Hypoxia-inducible factor 1- α (NCBI reference sequence NP_001521.1). The gene encoding PasB with 6 histidine codons attached at the 3' end was synthesized by Life Technologies and provided in a pMAT vector between *Nde*I and *Not*I restriction enzyme sites. As boundaries of a protein domain are not always easy to identify and to ensure the correct folding of rPasB domain, the encoding gene was extended with a few codons at both its N- and C- terminal ends to encompass amino acids 235–247 of Hypoxia-inducible factor 1- α [31]. After digestion with *Nde*I and *Not*I, the fragment was ligated into *Nde*I and *Not*I-digested pET21b vector, and then electro-transformed into *E. coli* BL21 (DE3) competent cells and plated on selective medium. To verify the construct, the cloned insert was amplified with the universal T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') and T7 terminator primer (5'-GCTAGTTATTGCTCAGCGG-3') in a PCR using a single colony to provide template DNA. The presence of the expected nucleotide sequence of the amplicon was confirmed. The clone was cultured in TB at 37 °C and expression of rPasB was induced with 1 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) (Duchefa Biochemie B.V., Netherlands) when cells reached the mid-logarithmic growth phase. Cells were further incubated overnight at 28 °C, before harvesting by low speed centrifugation. Pelleted cells were resuspended in PBS and cracked by sonication to release their cytoplasmic contents. The rPasB was purified by IMAC and SEC, and analyzed by SDS-PAGE and western blot.

2.3. Construction of a Nb library

A young dromedary (Canary Islands, Spain) was immunized with our rPasB preparation to raise an immune response. The animal received six subcutaneous injections at weekly intervals with 100 µg of the rPasB fraction, (mixed with complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent boosts). Three days after the last boost, 200 ml anti-coagulated blood was collected from the jugular vein. Lymphocytes were isolated from this anti-coagulated blood on Leucoprep™ tubes (Greiner Bio-One, Kremsmünster, Austria). Total RNA was purified from collected blood lymphocytes and used as template in a reverse transcription reaction for cDNA synthesis. Briefly, the cDNA was synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR) with oligo-dT₁₂₋₁₈ as primer. Then, the cDNA was used as template in a two-step PCR to amplify the DNA sequences encoding the VHH gene fragments. In the first step PCR, the sequence between the leader sequence and the CH2 region was amplified with primers CALL001 (5'-GTC CTG GCTGCT CTT CTA CAA GG-3') and CALL002 (5'-GGT ACG TGC TGT TGA ACT GTT CC-3'), respectively. Two main PCR products are obtained that are separated on 1% agarose gel. The shorter PCR fragment (700 bp) corresponding to the VHH-hinge-CH2 region of the HCAs was cut out of gel and extracted from agarose using the QIAquick Gel Extraction Kit (Qiagen, Germany) and amplified by nested PCR with primers A6E (5'-GAT GTG CAG CTG CAG GAG TCT GGR GGA GG-3') and PMCF (5'-CTA GTG CGG CCG CTG AGG AGA CGG TGA CCT GGG T-3') with *Pst*I and *Not*I restriction enzyme sites (underlined). After purification, the resulting VHH-PCR product was digested with *Pst*I and *Not*I restriction enzymes and the digested PCR fragments were ligated into the pMECS phagemid vector (cut with the same restriction enzymes) at 16 °C for 16 h, and electro-transformed into *E. coli* TG1 electro-competent cells (Lucigen, Middleton, USA). Immediately after the electro-shock, cells (aliquots of 0.05 ml) were diluted with 0.95 ml SOC medium per electroporation, pooled and incubated at 37 °C for 1 h. The transformed cells were then plated

on LB agar plates containing 2% glucose and 100 µg/ml ampicillin. While a diluted aliquot of cells was plated on standard-sized Petri dishes to determine the total amount of transformants, the majority of the cells were loaded on large Petri dishes. After overnight incubation at 37 °C, colonies of TG1 cells were scraped from the large plates and stored in LB medium with 20% glycerol in aliquots of 1 ml. Assessment of the percentage of clones with a phagemid containing an insert with the proper size of a VHH gene was performed by colony PCR with MP57 and GIII primers on 95 randomly chosen colonies. The MP57 (5'-TTA TGC TTC CGG CTC GTA TG-3') and GIII (5'-CCA CAG ACA GCC CTC ATA G-3') primers anneal on the vector sequences abutting the cloned VHH insert.

2.4. Display of Nbs on phage particles and biopanning

The Nbs from the immune library were expressed on phage particles using VCSM13 helper phages. Antigen-specific Nbs were enriched from this phage displayed Nb bank by four consecutive rounds of *in vitro* selection. An aliquot (1 ml) *E. coli* TG1 cells containing the Nb library was grown in 300 ml 2×TY medium with 1% (w/v) glucose and 100 µg/ml ampicillin until an OD₆₀₀ value of 0.6–0.9 was reached. The *E. coli* cells were subsequently infected with ~10¹² VCSM13 helper phages for 30 min at room temperature (RT). The infected cells were harvested by centrifugation at 2700 rpm for 10 min before resuspending the cell pellet in 300 ml fresh 2×TY medium supplemented with 100 µg/ml ampicillin and 70 µg/ml kanamycin to produce virions with Nbs at their tip. After overnight incubation, bacterial cells were removed by centrifugation and the virus particles in the culture supernatant were precipitated with polyethylene glycol (PEG)/NaCl and resuspended in a total volume of 1 ml sterile PBS.

For biopanning, 100 µl of rPasB protein mixture (100 µg/ml) was coated overnight at 4 °C, in one well of a microtiter plate. Residual protein binding sites in this rPasB-coated well and in another well at a distant location were blocked with either 4% skimmed-milk proteins, Protein free buffer (Thermo Scientific), 0.1% Casein or SEA BLOCK blocking buffer (Thermo Scientific), used during the sequential rounds of biopanning, in an attempt to minimize phage enrichment by binding to components within the 'blocking' reagent. Phage particles (~10¹¹ plaque forming units) were added to the wells and incubated for 1 h at room temperature. After removing non-adsorbed phages by multiple washings with PBST (PBS containing 0.05% Tween-20), phage particles bound to the rPasB-coated wells were eluted with 100 µl triethylamine (100 mM TEA, pH 11.0) for 10 min. The released particles were immediately neutralized with 100 µl Tris-HCl (1.0 M, pH 7.4). An aliquot of 10 µl of the released phage particles from each well were serially diluted (10 fold) and 10 µl added to a fixed amount (90 µl) of TG1 cells (in exponentially growth phase). After 30 min incubation, at RT to allow infection, a 10 µl aliquot of each dilution was striped on agar plates with selective medium and incubated overnight at 37 °C to assess relative phage enrichment between rPasB coated and non-coated wells. The remaining fraction of the rPasB-eluted phages was used to infect exponential growing *E. coli* TG1 cells to produce phage particles for the next round of panning. The panning was performed for 4 consecutive rounds of selection [32].

2.5. Screening of specific Nbs by ELISA

Individual colonies were randomly picked and grown in 100 µl of 2×TY medium supplemented with 10% glycerol, 2% glucose and 100 µg/ml ampicillin in a 96 round bottom well plate. After overnight incubation at 37 °C, 10 µl of each of these *E. coli* cultures were transferred to a 2 ml deep well plate to inoculate 1 ml 2×TY supplemented with 0.1% glucose and 100 µg/ml ampicillin. The

remainder of the overnight culture was frozen at –20 °C and kept as a master plate. After 4–5 h incubation (shaken at 37 °C) of the deep well plate cultures, 1 mM IPTG was added to induce expression of the encoded Nbs with HA and His-tags. After another 4 h incubation the protein extract of these cells were obtained by freeze-thaw cycles and used in an ELISA to assess the capacity of the Nbs to recognize coated rPasB protein in a 96-well microtiter plate. Each soluble extract was added to a well coated with 100 µl rPasB at 2 µg/ml and another well without rPasB, which was used as negative control. The antigen bound Nbs were detected with 1/2000 diluted mouse anti-HA IgG and 1/2000 goat anti-mouse IgG conjugated with Alkaline Phosphatase (ImTec Diagnostics NV, Antwerp, Belgium). The colonies, corresponding to the extracts that showed positive signals in ELISA (at least 2-fold higher compared with the background signal from a well without antigen) were grown and used to prepare phagemid DNA, from which the Nb insert was sequenced and analyzed.

2.6. Expression and purification of selected Nbs

The pMECS plasmids containing the genes of the clones that scored positive in ELISA and that belong to different Nb families were extracted from TG1 cells, electro-transformed into *E. coli* WK6 cells and plated on agar plates with selective medium. Single colonies were picked for expression of Nbs fused with HA- and His-tags at their C-terminus. The WK6 cells were cultured in shake flasks by growing the bacteria in 330 ml of TB medium supplemented with 0.1% (w/v) glucose, 100 µg/ml ampicillin and 2 mM MgCl₂ until an optical density at 600 nm between 0.6 and 0.9 was reached. Nb expression was then induced with 1 mM IPTG for 16 h at 28 °C. After pelleting the cells by low speed centrifugation, recombinant Nbs produced in the periplasm were released by osmotic shock [33] and loaded on a column with Ni-NTA resin (ThermoScientific). After washing off non adsorbed material, the retained proteins were eluted from the resin with 500 mM imidazole and the fractions desalted by gel filtration on Superdex™ 75 10/300 GL (GE Healthcare). The purity and identity of the protein in the fractions were evaluated by SDS-PAGE stained with Coomassie and western blot with 1/2000 diluted mouse anti-His antibody as primary antibody and a goat anti-mouse IgG conjugated with HRP, diluted 1/2000, as secondary antibody. The final protein yield was determined from the UV absorption at 280 nm, using the theoretical extinction coefficient of the Nb as calculated from its amino acid content. Ultimately, the purified specific Nbs were brought to 1 mg/ml in PBS for storage.

2.7. Antigen specificity of Nbs

Western blot was employed to verify the antigen specificity of the Nbs. In total, 2 µg of purified rPasB was loaded onto a NuPAGE 10% Bis-Tris protein gel (ThermoScientific) by using a one-slot comb gel. The separated proteins were transferred to a nitrocellulose membrane. Subsequently, residual protein binding sites on the membranes were blocked with 5% skimmed milk PBS. The membrane was cut in strips and each strip was incubated separately with a Nb (1 µg/ml) in sterile PBS at 4 °C. Next, the membranes were washed in PBST and incubated, first with a mouse anti-HA monoclonal antibody and subsequently with an HRP-conjugated goat anti-mouse monoclonal antibody. As a positive control, an HRP conjugated mouse anti-His monoclonal antibody was used. A Nb directed against an unrelated antigen was used as a negative control. The binding of Nbs against endogenous proteins from *E. coli* strains was evaluated by loading cell lysates of BL21 (DE3), Shuffle T7 and WK6 *E. coli* onto a protein gel, transferred to membranes and then incubated with the different Nbs at RT. The endogenous

proteins from lysates of human HEK293T and HeLa tumor cells were used to test the reactivity of our Nbs towards proteins in mammalian cell lines, following the protocol as described for *E. coli* lysates.

2.8. Identification of the contaminant protein by LC-MS/MS

The contaminant protein in the rPasB preparation was immunocaptured with a specific Nb and then loaded onto a NuPAGE 10% Bis-Tris protein gel. After electrophoresis, Coomassie staining was performed to visualize the protein bands. After complete destaining of the background, the band of the contaminant was excised with a razor blade and placed into a sterile 1.5 ml microcentrifuge tube. The protein was then digested with trypsin and the generated peptides were analyzed by LC-MS/MS. For peptide identification, peak lists were generated using the application spectrum selector in the Proteome Discoverer 1.4 package. The resulting peak lists were searched using Sequest against an *E. coli* protein database obtained from Uniprot. Peptide matches were filtered using Percolator within Proteome Discoverer and manually validated. Probability was calculated by the Percolator algorithm ensuring an estimated false detection rate (FDR) below 5%.

2.9. Characterization of Nbs

2.9.1. Thermal stability of Nbs

Thermal stability of the purified Nbs was measured by thermofluorescence in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Purified Nbs were concentrated in Vivaspin® 2 ml Concentrators (Sartorius) to a final concentration of 2.5 mg/ml. Subsequently, 15 µl of the Nb protein was mixed with 5 µl of 1/200 diluted SYPRO® Orange Protein Gel Stain (ThermoScientific) and 10 µl of sterile PBS to reach a final volume of 30 µl. Triplicates were prepared for each Nb and three wells containing only PBS and SYPRO® Orange Protein Gel Stain were used to generate the baseline control. The temperature was increased from 25 to 95 °C at a rate of 0.5 °C/min. The data was analyzed in Origin using non-linear fitting to determine the melting temperature (T_m) of the Nbs.

2.9.2. Affinity of Nbs to antigen

The affinity of Nbs for their antigen was estimated in ELISA. Wells of microtiter plates were coated overnight at 4 °C with 100 µl of the rPasB sample at 0.2 and 2 µg/ml. Later these wells were 'overcoated' with 3% skimmed milk (1 h at room temperature) to block residual protein binding sites and one additional row of wells was coated with 3% skimmed milk to serve as a negative control. The serial diluted Nbs were added at concentrations of 1000, 100, 50, 10, 5, 1, 0.1 nM (corresponding to 14, 1.4, 0.7, 0.14, 0.07, 0.014, 0.0014 µg/ml, respectively) and incubated for 1 h at room temperature. After washing, the wells were filled with 100 µl of a 1/2000 diluted mouse anti-HA monoclonal antibody. Subsequently, 1/2000 diluted HRP conjugated anti-mouse IgG was incubated as the secondary antibody and TMB was used as substrate. The equilibrium dissociation constant (K_D) of the Nbs to the antigen was estimated as the concentration of Nb that yielded half of the maximal signal.

2.10. In vivo biotinylation of specific Nb

Considering the production yield and the affinity of the Nbs, Nb5 was chosen to generate a biotinylated format. Briefly, the Nb5 encoding gene was subcloned into the vector pBAD17 (this plasmid confers resistance to ampicillin; the nanobody will be expressed from the lac promoter and will contain at its C-terminal end the IgA hinge and the AVI-tag for *in vivo* biotinylation [34] by BirA) and the

recombinant plasmid was electro-transformed into WK6 competent cells together with the plasmid pBirA (containing chloramphenicol resistance gene and the gene for BirA enzyme under control of a lac promoter). The transformed clones harboring both plasmids were selected on LB agar plates supplemented with ampicillin and chloramphenicol. The clone was cultured in 330 ml of TB medium and 330 µl of 50 mM biotin was added when an OD_{600nm} value of 0.4 was reached. The expression of biotinylated Nb5 (Bio-Nb5) was induced with 1 mM IPTG, overnight at 28 °C. Next, the periplasmic proteins containing Bio-Nb5 were released by osmotic shock. The Bio-Nb5 was purified through a Streptavidin-Mutein Matrix (10 ml, 50% suspension, Roche) according to the protocol provided by the manufacturer using an elution buffer containing 4 mM biotin for competition binding. The residual biotin molecules were removed by SEC and the purified Bio-Nb5 samples were stored in sterile PBS at –80 °C. The concentration of the protein was determined by Nanodrop at 280 nm. Purity and biotinylation of Bio-Nb5 was confirmed by Coomassie stained SDS-PAGE and by western blot using an HRP conjugated streptavidin probe.

2.11. Strategies to remove contaminant protein from rPasB samples

2.11.1. Immunoprecipitation of contaminant protein by Nb5

The strategy to remove the contaminant protein from our rPasB sample with Nb5 is shown schematically in Fig. 1. To form the immunocomplex, 200 µl of the rPasB sample (estimated at 0.25 mg/ml and contaminated with SlyD) was incubated overnight at 4 °C with 20 µg Nb5. Simultaneously, 50 µg mouse anti-HA monoclonal antibody was mixed with 30 µl Protein A/G plus-agarose beads (Santa Cruz Biotech) in 200 µl immunoprecipitation buffer (IPB: 40 mM Tris-HCl pH 8.0, 1% Triton, 10% glycerol, 280 mM NaCl supplemented with 1/1000 protease inhibitor cocktail (1000× Leu pepsin at 1 mg/ml, AEBSF at 30 mg/ml, EDTA at 100 mM)) by turning head-over-head, overnight at 4 °C. Antibody-coated Protein A/G beads were then blocked with 1% BSA in IPB at 4 °C for 30 min and washed once with IPB buffer. Next, the beads were added to Nb5 pre-incubated with the recombinant protein sample and the mixture was incubated again, turning head-over-head, overnight at 4 °C. Beads were pelleted by centrifugation and the cleared supernatant was saved. Beads were washed 10 times with IPB, resuspended in a final volume of 60 µl water with 4× NuPAGE LDS sample buffer and 10× NuPAGE sample reducing agent (ThermoScientific) and heated at 90 °C for 5 min. The immunocomplex fraction was loaded onto a NuPAGE 10% Bis-Tris protein gel (ThermoScientific) to separate the proteins. Proteins were visualized by silver staining and by western blot using first Nb5, followed by mouse anti-HA monoclonal antibody and HRP-conjugated goat anti-mouse IgG antibodies.

2.11.2. Clearance of contaminant protein by treating with Bio-Nb5

Since SEC fails to separate the contaminant SlyD protein from our rPasB sample, we decided to increase the MW of this contaminating protein by complexation with Bio-Nb5, thereby increasing the elution volume in SEC between the rPasB peak and the peak from the complex, Bio-Nb5::SlyD. Fig. 2 shows a schematic representation of the strategy to remove the contaminating protein from the rPasB protein sample. First, we cultured BL21 (DE3) cells with the rPasB expression plasmid and induced expression of rPasB protein with 1 mM IPTG, overnight. The cells were collected and lysed by sonication. The cell lysate was loaded onto a Ni-NTA column for affinity chromatography. After thorough washing, the bound proteins were eluted from the column with 0.5 M imidazole in PBS. This fraction was applied on Superdex™ 75 10/300 GL (GE Healthcare). The broad peak of rPasB (contaminated with SlyD) was

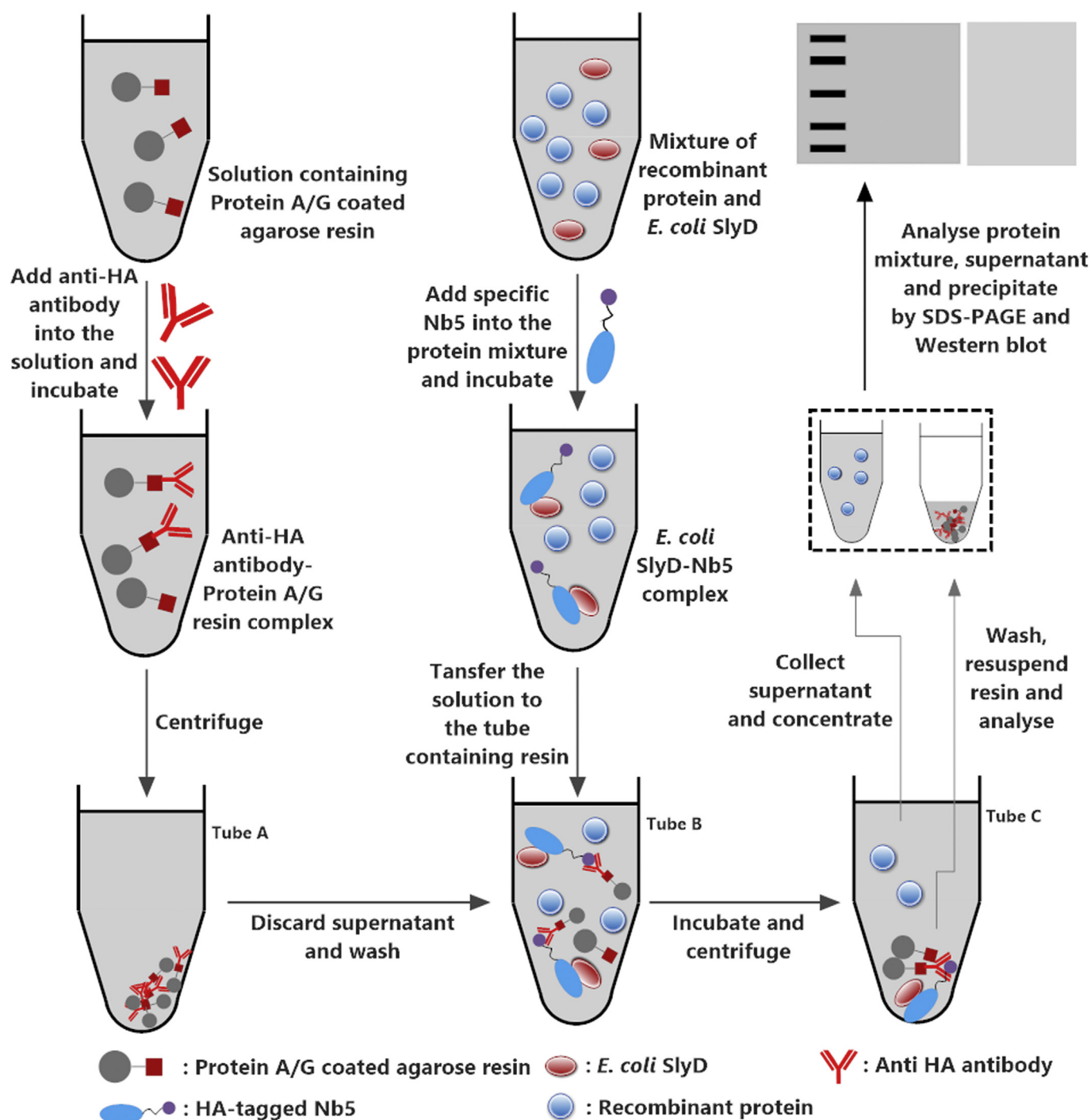


Fig. 1. Schematic representation of the strategy to eliminate *E. coli* SlyD from recombinant protein samples with Nb5 via immunoprecipitation. Excess Protein A/G agarose beads are incubated with mouse anti-HA monoclonal antibodies to form the anti-HA Ab-Protein A/G resin complex. After centrifugation and removal of the supernatant, the insoluble complex from Tube A is used to capture the Nb5 via its HA tag. In parallel, the rPasB sample contaminated with SlyD (as obtained after IMAC and SEC) is incubated with HA-tagged Nb5, to form SlyD::Nb5 complex in Tube B. The insoluble complex from Tube A is transferred to Tube B to allow the binding between the anti-HA antibody with the HA-tagged Nb5 (Tube C). The supernatant obtained after centrifugation of Tube C, which contains rPasB is transferred to a fresh tube for further use. The pellet in Tube C is washed to reduce non-specific binding on the resin. The protein contents within the supernatant and the immunocomplex (washed pellet) are analyzed by SDS-PAGE and western blot using HA-tag specific antibodies.

taken and mixed with Bio-Nb5 (in molar excess over SlyD) and incubated for 2 h at 4 °C to enable the binding between Bio-Nb5 and the contaminating SlyD protein. The mixture was concentrated to an appropriate volume for injection onto the gel filtration column. Different peaks eluting from the SEC were collected and analyzed by SDS-PAGE to determine their content. The excess of

Bio-Nb5 in the rPasB fraction was removed by an additional IMAC. The Bio-Nb5 remains in the flow through, as it doesn't contain a His-tag while the rPasB domain can be eluted with imidazole as described earlier. The imidazole in the eluted fraction is eliminated by gel filtration. The purity of rPasB protein was checked by SDS-PAGE.

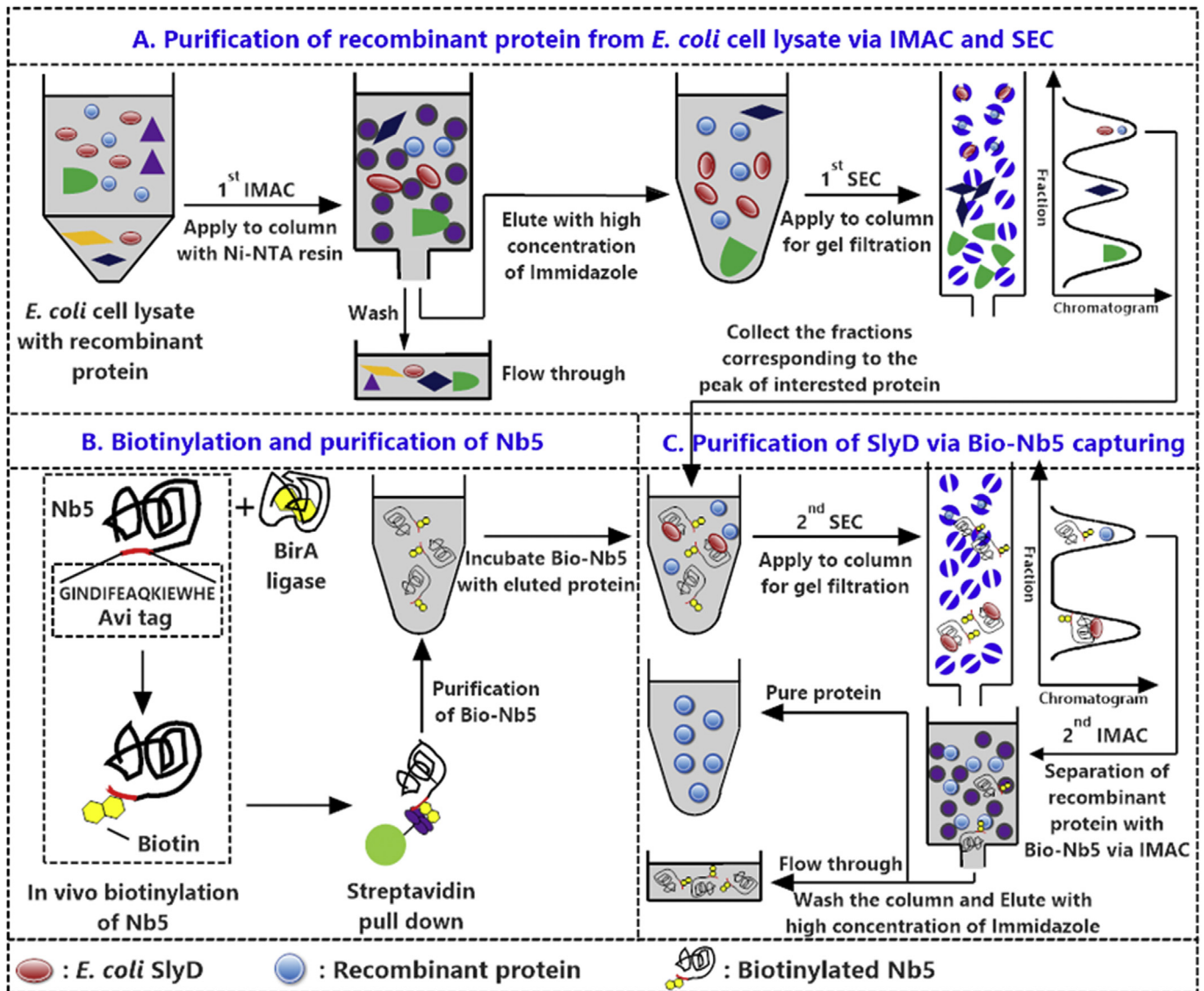


Fig. 2. Schematic representation of the strategy to eliminate *E. coli* SlyD from recombinant protein samples by complex formation with Bio-Nb5 and separation via SEC. (A) The rPasB protein is enriched from an *E. coli* BL21(DE3) cell lysate by IMAC and desalted by SEC. After overnight induction with IPTG, BL21(DE3) cells expressing rPasB in their cytoplasm are collected and lysed by sonication. The cell lysate is loaded onto a Ni-NTA column. After washing off the non-binding components (flow-through fraction), the Ni-NTA captured proteins are eluted from the column with imidazole. Then, these proteins are desalted by SEC and the fractions eluting at the expected MW of the recombinant protein (rPasB) are collected. (B) *In vivo* biotinylation and purification of Bio-Nb5. WK6 cells co-transformed with the pBAD17 plasmid, containing the gene for Nb5, a hinge and the AVI-tag, and the pBirA plasmid, which encodes BirA enzyme under control of a lac promoter. After adding Biotin to the culture and inducing the protein expression with IPTG, the *in vivo* biotinylated Nb5 is purified on Streptavidin-Mutectin Matrix (excess biotin is removed from Bio-Nb5 by SEC). (C) Removal of *E. coli* SlyD by SEC after complexing with Bio-Nb5. Bio-Nb5 (from B) is added in excess to SlyD contaminated rPasB sample (from A) and incubated to form Bio-Nb5::SlyD complex. Then, SEC separates the free and complexed proteins from the mixture. The Bio-Nb5::SlyD complex (eluting in a first peak) is separated from free Bio-Nb5 and rPasB, which elute together in a second peak. Finally, the Bio-Nb5 and rPasB mixture is passed over a Ni-NTA resin. The flow-through contains the free Bio-Nb5 and the pure rPasB is eluted with imidazole. (Alternatively, passing the Bio-Nb5 and rPasB mixture over Streptavidin containing columns can be used to capture the Bio-Nb5 and to obtain pure rPasB protein in the flow-through).

3. Results

3.1. PasB gene construction

The gene of the PasB antigen was synthesized and cloned into the pET21b vector between the restriction enzyme sites *Nde*I and *Not*I. The cloned fragment is under control of the T7 promoter, and upon induction, the PasB protein will be produced with a His-tag at its C-terminus to facilitate purification by IMAC. The nucleotide sequence of the cloned insert showed a 100% identity to the designed gene. The cytoplasmic extract of the induced culture was purified through IMAC and SEC. Increasing amounts of the SEC

fraction eluting at a MW around 15,000 were separated by SDS-PAGE and stained with Coomassie to assess the purity of the rPasB protein (Fig. 3). The rPasB band of 14,000 is clearly present, however, with 5 μ g applied on gel, one band around MW of 28,000 is also visible, which accounts for less than 1 μ g (estimated from intensity of loading 1 μ g on the same gel). Western blot of the same sample separated by SDS-PAGE under reducing and non-reducing conditions confirmed that only the band with MW of 14,000 contained the expected His-tag of the recombinant protein. The absence of the band of MW 28,000 in western blot, indicates that this band corresponds to an independent bacterial protein co-purifying with the rPasB during IMAC and SEC, rather than e.g.

dimerized rPasB polypeptides. Nevertheless, this fraction of rPasB with the contaminant was used to immunize a dromedary and to generate Nbs.

3.2. Construction of immune Nb library, phage display and panning

A young dromedary was immunized with the rPasB preparation to raise an immune response in its heavy chain-only antibodies. The blood of the immunized dromedary was collected from the jugular vein and the immune Nb library was constructed in our pMECS phage display vector. The size of the Nb library in *E. coli* TG1 cells comprised about 3.3×10^8 individual transformants. A colony PCR on 95 randomly chosen individual colonies was performed and the amplicons separated by electrophoresis on agarose gel. An amplicon of around 700 bp is expected for clones with a phagemid carrying a Nb gene. From the agarose gel electrophoresis it was estimated that about 77% of the colonies within our library possess phagemids with the correct insert size of a Nb gene (the PCR of 73 out of 95 clones yielded an amplicon of 700 bp).

A representative aliquot of the immune Nb library was cultured and infected with VCSM13 helper phages to express the Nb at the tip of M13 virion particles. This phage displayed Nb library was used for biopanning on immobilized rPasB antigen. The ratio of infective phage particles eluted from wells with and without rPasB antigen during four consecutive rounds increased from 1.1 to 42.

3.3. Screening for antigen-specific Nbs with bacterial extracts in ELISA

To screen for presence of Nbs recognizing the antigen used to immunize, we randomly picked 190 individual colonies from the

2nd (32 colonies), 3rd (63 colonies) and 4th (95 colonies) round of panning. The extracts from these 190 cultures, induced with IPTG to express Nbs (fused with the HA-tag and His-tag at their C-terminus) were tested in ELISA in wells coated with or without the rPasB sample. The ELISA revealed that 53 extracts had a signal strength that was at least two fold higher than the signal of control wells containing the same extract but without coated rPasB sample. Colony PCR was performed on these 53 clones and their Nb gene sequences were determined. After analyzing the amino acid sequence from the *in silico* converted Nb nucleotide sequence, we confirmed the presence of 7 unique Nb families, represented by Nb3, Nb5, Nb12, Nb21, Nb23, Nb35 and Nb127. The aligned Nb amino acid sequences, numbered according to the 'International Immunogenetics Information System' (IMGT) are shown in [supplementary material](#). As expected, the highest sequence variability among these clones is noted in the CDR3. The results of an ELISA with these 7 purified Nbs gave a rapid and high signal for clones Nb23 and Nb35. The signal with the other Nbs needed longer incubation times with substrate.

3.4. Expression and purification of antigen specific Nbs

To obtain the Nbs without gene 3p fusion we transformed the pMECS plasmids containing the Nbs of interest into WK6 cells. WK6 cells cannot suppress the amber stop codon between the tags downstream of the Nb gene and the gene 3, and these cells are therefore forced to produce monomeric Nbs with a HA-tag and a His-tag at their C-terminus. Soluble Nbs were extracted from the periplasm by osmotic shock and purified by Ni-NTA affinity chromatography and SEC. SDS-PAGE of the purified Nbs stained by Coomassie revealed the presence of one single band with MW of

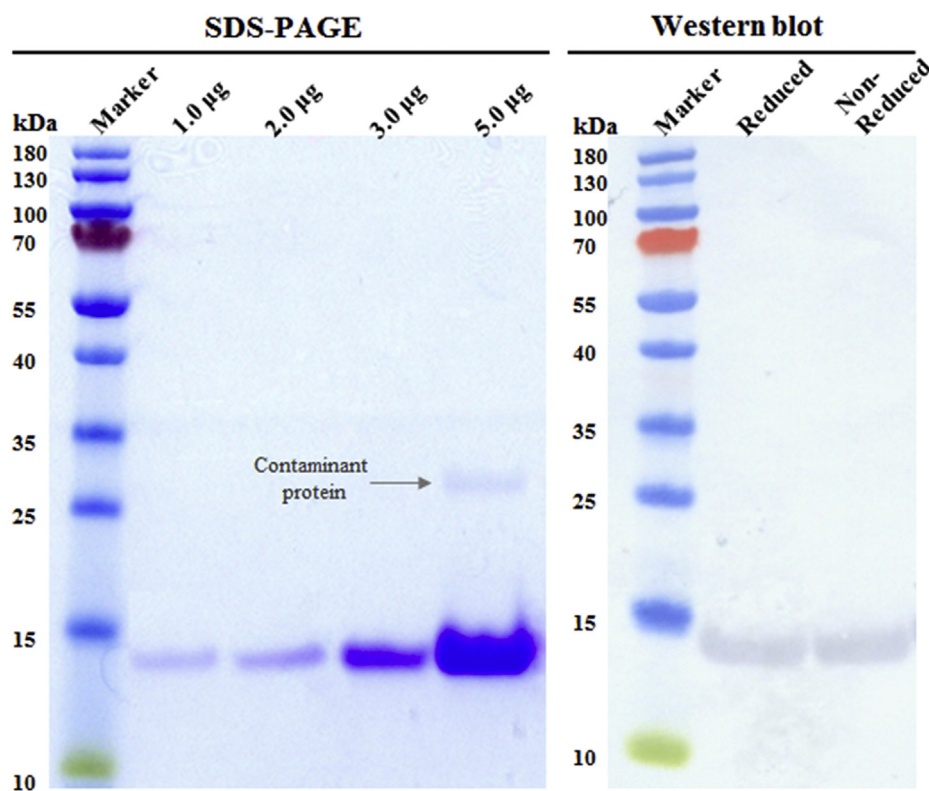


Fig. 3. Identification and purity of rPasB assessed by SDS-PAGE, where proteins are revealed with Coomassie staining or western blot. Different quantities (estimated at 1 µg, 2 µg, 3 µg and 5 µg) of rPasB preparation, as obtained after purification by IMAC and SEC, were loaded onto the gel and stained with Coomassie (left) or western blot using an anti-His monoclonal antibody (right).

around 14,000 as expected from their amino acid sequence and it demonstrated their purity. The absence of dimerized Nb formats and the presence of a His-tag on these Nbs was confirmed by western blot.

3.5. Target specificity of the Nbs

The antigen-specificity of the recombinant Nbs was analyzed by western blot after separating our rPasB sample on SDS-PAGE under reducing and non-reducing conditions (Fig. 4). After blotting the proteins to the membrane and incubating a strip of this membrane separately with the 7 Nbs we detected the presence of Nbs with HA-specific monoclonal antibodies. The result demonstrated unequivocally that Nb23 and Nb35 target rPasB, as confirmed by the band observed with MW 14,000. Remarkably, the remaining 5 Nbs target the contaminating protein of MW 28,000, pointing towards the immunodominance of this protein during immunization. To direct the immune response solely against the protein of interest and to avoid a high titer against contaminants, it seems crucial to eliminate this immunodominant bacterial protein from the recombinant immunogen. Therefore, we aimed to identify the contaminant and develop a strategy to eliminate it in from future recombinant protein preparations before immunizing dromedaries or llama's.

3.6. Identification of contaminant protein by LC-MS/MS

Nb5 was used to immunocapture the contaminating protein from our rPasB sample. After separation by SDS-PAGE, we excised the band of interest and digested the protein with trypsin. The tryptic digest was analyzed by LC-MS/MS and a search of the peptides was performed against the Uniprot database using Sequest. As shown in Table 1, the search identified the contaminant protein as *E. coli* SlyD. This protein has been reported to be a potential common contaminant appearing after purification of recombinant proteins via IMAC [19,35]. To confirm the bacterial

origin of this protein and its natural occurrence in various *E. coli* strains, we prepared bacterial lysates of multiple *E. coli* strains, separated the contents of the lysates by SDS-PAGE and performed a western blot using Nb5 and an HA-specific monoclonal antibody to reveal the HA-tag of the Nb. Fig. 5 confirms the presence of a Nb5-specific band with MW 28,000 in all tested *E. coli* strains, whereas no band is observed with lysates from human HeLa or HEK293T mammalian cell lines.

3.7. Characteristics of Nbs binding to SlyD

3.7.1. Thermal stability

The thermal stability of the five SlyD-specific Nbs was monitored by thermofluorescence in a CFX Connect™ Real-Time PCR Detection System. A high thermal stability was observed for all five Nbs, with T_m -values ranging from 57 °C to 67 °C (Nb5 has an intermediate T_m of 60.7 °C $\pm$ 0.1).

3.7.2. Affinity of Nbs for SlyD

The affinity of the five selected Nbs towards SlyD was estimated via saturation ELISA binding studies against immobilized antigen. The Nbs were serially diluted and added at concentrations ranging from 1000 nM to 0.1 nM. The observed apparent affinities were recorded at 1.5 nM, 2.5 nM and 4.5 nM for Nb5, Nb12 and Nb21, respectively. A lower affinity was observed for Nb3 and Nb127 with values of 32 and 25 nM, respectively. Since Nb5 shows the best apparent affinity, we selected this Nb as candidate to develop an affinity adsorbent to eliminate SlyD from recombinant *E. coli* protein extracts.

3.8. Expression and purification of biotinylated Nb

The Nb5 gene was subcloned into the pBAD vector for over-expression and *in vivo* biotinylation in WK6 cells containing the plasmid pBirA. The biotinylated Nb5, i.e., Bio-Nb5, was extracted from the periplasm and purified with a Streptavidin-Mutein Matrix

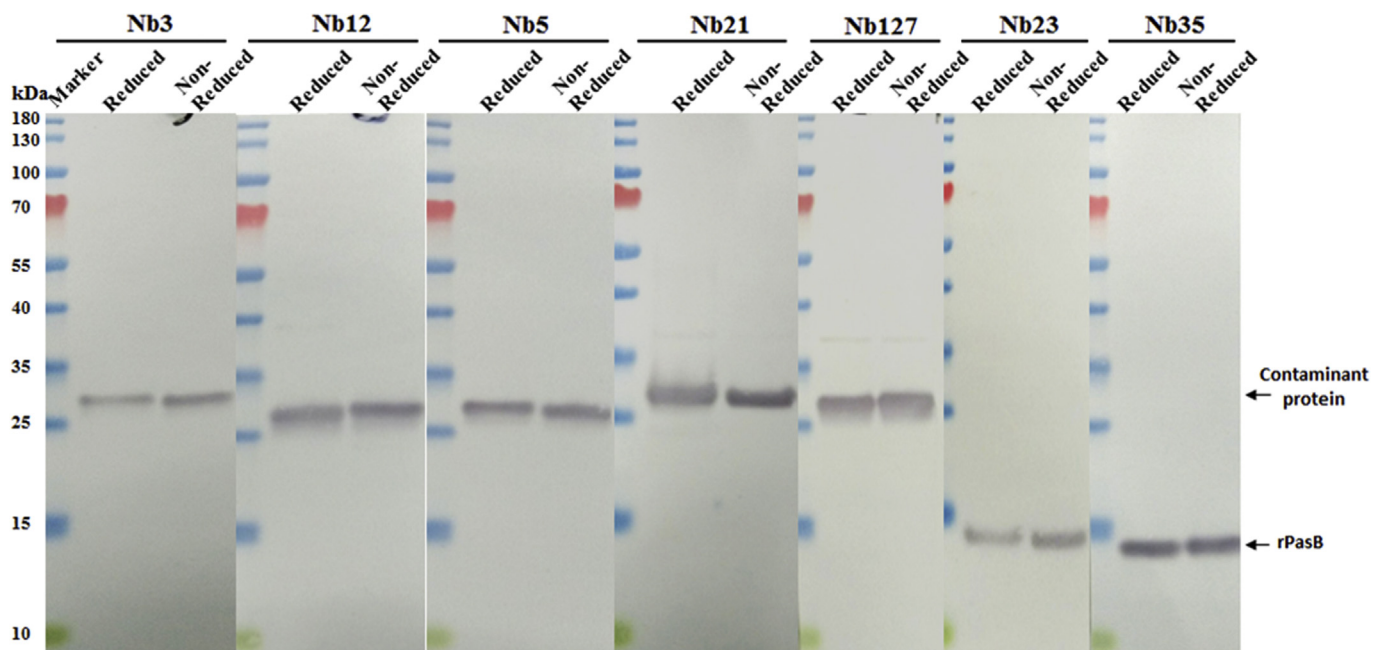


Fig. 4. Antigen-specificity of the Nbs. An rPasB sample contaminated with a protein (that was later determined to be SlyD) was separated by SDS-PAGE under reducing and non-reducing conditions and transferred to a nitrocellulose membrane. Different strips were incubated with the Nbs and the presence of the Nb was detected with monoclonal anti-HA antibodies.

Table 1
Identification by LC-MS/MS of the protein contaminating the rPASB sample. Contaminant protein is captured by Nb5, separated by SDS-PAGE and digested with trypsin before LC-MS/MS analysis.

Sequence	PSMs	Protein groups	Protein group accessions	PEP	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]
FLAETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLK	4	1	P0A9L1	0.00005909	4.38	3	3859.73581	−145.65	62.52
DVFMGVDELQVGMR	1	1	P0A9L1	0.001518	4.26	2	1597.55290	426.35	60.56
FDVAVGANDAYGQYDENLVQR	5	1	P0A9L1	0.02437	4.09	2	2347.32207	780.70	56.03
FNVEVVAIR	8	1	P0A9L1	0.06429	3.34	2	1048.11516	830.90	54.27
FLAETDQGPVPVEITAVEDDHVVVDGNHmLAGQNLK	1	1	P0A9L1	0.01087	3.14	3	3875.30564	−255.92	59.21
DVFmGVDELQVGMR	2	1	P0A9L1	0.002358	3.01	2	1612.04546	−512.23	60.53
DLVVSLAYQVR	1	2	P0A9L1; Q7CFU4	0.04346	2.64	2	1263.26445	−171.74	57.53
DVFmGVDELQVGmR	3	1	P0A9L1	0.01186	2.60	2	1628.42436	−274.02	56.48
Identification FKBP-type peptidyl-prolyl cis-trans isomerase SlyD from <i>Escherichia coli</i> O157:H7									

PSMs: peptide sequence matches.
PEP: Posterior error probability.
MH+ [Da]: protonated molecular weight.
ΔM [ppm]: mass shift.
RT [min]: Retention time.

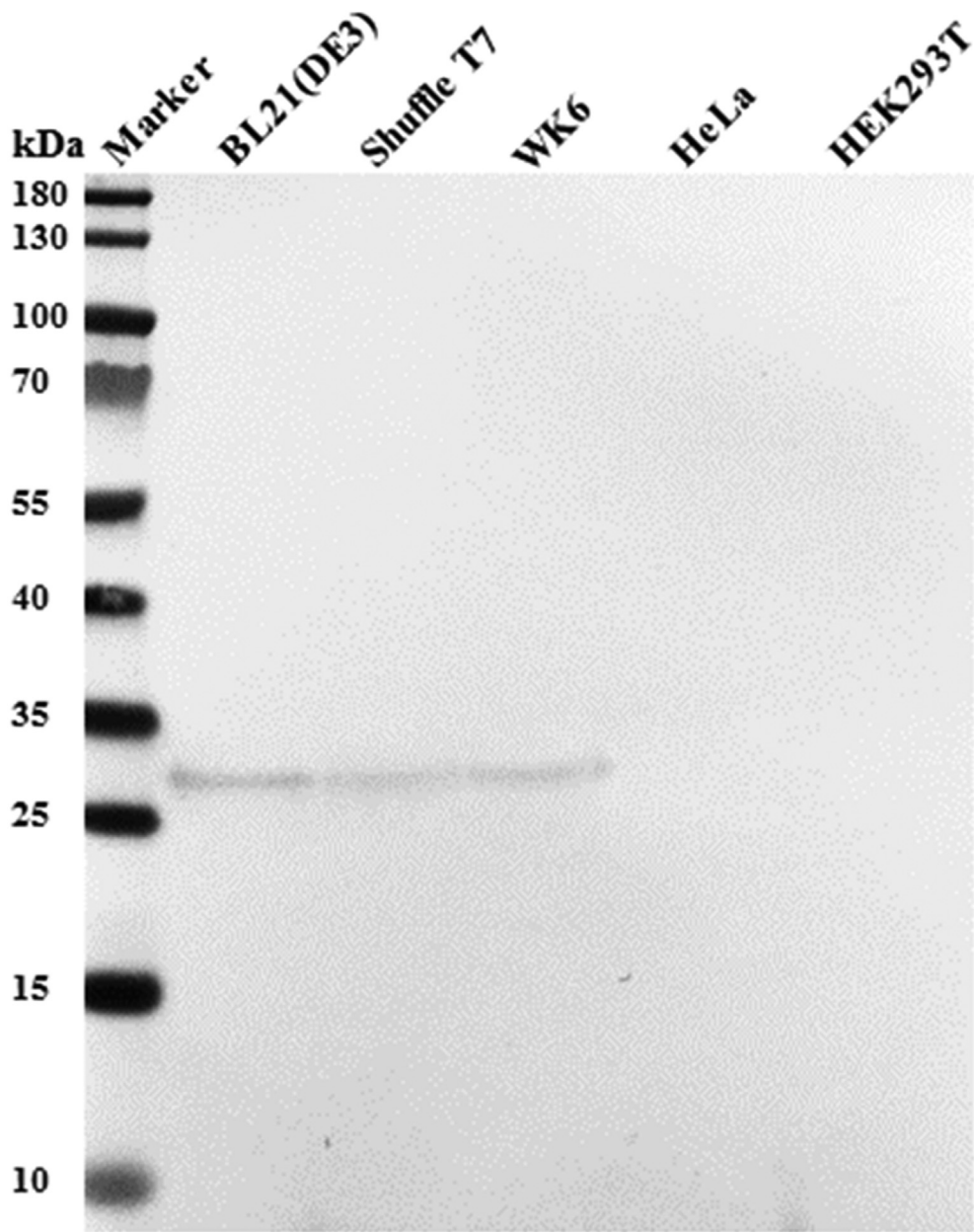


Fig. 5. Occurrence of SlyD recognized by Nb5 in lysates of various *E. coli* strains and absence of cross-reactive proteins in mammalian HeLa and HEK2937T cell lysates.

according to the manufacturer's instructions. An SDS-PAGE of the purified Bio-Nb5 stained by Coomassie revealed the presence of one single band with MW of 20 kDa, as expected from its amino acid sequence (including the Nb, the IgA hinge and the biotinylated AVI tag). This Bio-Nb5 preparation was very pure and its successful *in vivo* biotinylation was confirmed by western blot using streptavidin-HRP conjugate as a probe. Furthermore, its binding activity to *E. coli* SlyD protein was also confirmed by western blot with the streptavidin-HRP conjugate.

3.9. Removal of bacterial SlyD protein from rPasB sample by immunocomplexation with Nb5

3.9.1. Immunocapturing SlyD with Nb5

To check whether HA-tagged Nb5 can be used to capture *E. coli* SlyD from recombinant protein samples, we started with a heavily contaminated rPasB fraction (Fig. 6). We first added an excess of Nb5 (with HA- and His-tag) to form a complex with the contaminating *E. coli* SlyD. Subsequently, protein A/G agarose beads decorated with anti-HA monoclonal antibodies were added into the solution to capture Nb5 via its HA-tag. After centrifugation and washing of the beads, SDS-PAGE was performed to assess the purity of the rPasB protein after immunocapturing SlyD. Western blot was also performed to reveal the presence of the contaminant *E. coli* SlyD in the bead fraction (Fig. 6). Three bands, corresponding to Nb5, rPasB and *E. coli* SlyD could be revealed in the immunocomplex mixture. However, only one single band corresponding to the rPasB protein remained in solution after immunocapturing of the SlyD5, and as expected no residual contaminant *E. coli* SlyD stayed in the supernatant after immunoprecipitation with Nb5 (Fig. 6). The

result proves the efficacy of Nb5 to capture and eliminate efficiently *E. coli* SlyD from contaminated recombinant proteins. The yield of rPAS after removal of SlyD is given in Table 2. An overall recovery of around 50% was obtained following this purification strategy.

3.9.2. Removal of SlyD by complexation with Bio-Nb5 and gel filtration

After sonication, the rPasB protein was released from the cytoplasm of *E. coli* together with native SlyD protein. The cell lysate was loaded on a Ni-NTA column and SEC for purification of the His-tagged recombinant protein. After these purification steps, Bio-Nb5 was added to associate with *E. coli* SlyD. The Bio-Nb5::SlyD complex will be three times the size of our rPasB, which is expected to facilitate the separation of SlyD::Bio-Nb5 complex from rPasB by SEC. This was indeed, demonstrated, as the rPasB protein (peak III) was completely separated from the SlyD containing fractions (Fig. 7A). However, the excess of Bio-Nb5 is now contaminating the rPasB protein (Fig. 7B). For immunization in a dromedary this would not pose problems, as Nb5 will not be immunogenic in these animals. Nevertheless, the contaminating Bio-Nb5 can still be removed through a second IMAC, as it will elute in the flow-through since it doesn't carry a His-tag. The imidazole eluate of the second IMAC contains therefore only pure rPasB protein (Fig. 7C). A simple dialysis or gel filtration can be used to remove the imidazole from the pure rPasB. From Table 2 it can be seen that the recovery of pure rPasB is lower according to this purification strategy, however, this is due to the additional steps that were introduced to remove the excess of Bio-Nb5.

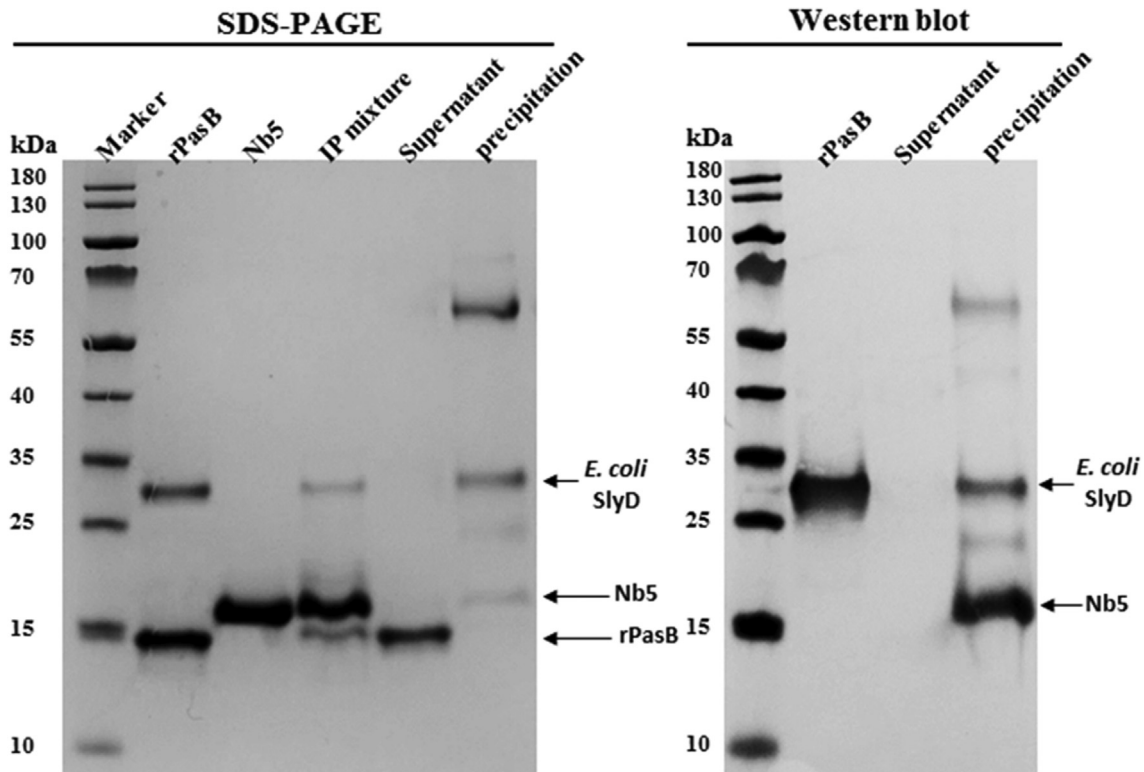


Fig. 6. Removal of SlyD contaminant from recombinant protein preparations after immunoprecipitation with Nb5. Lane labeled rPasB is the starting rPasB sample contaminated with SlyD. This preparation was mixed with excess Nb5 (pure Nb5 is shown in lane labeled Nb5) and applied on the lane 'IP mixture'. After adding Protein A/G beads decorated with HA-specific monoclonal antibodies and centrifugation, the supernatant is loaded on the lane 'Supernatant' and the pelleted beads after washings are applied on the lane 'precipitation'. The proteins from all samples were separated by SDS-PAGE and stained with Silver (left) or via western blot using Nb5, anti-HA antibodies and goat anti mouse IgG-HRP conjugate (right). The weak bands with MW 24,000 and 60,000 are the light and heavy chain of the anti-HA monoclonal antibody used to decorate the Protein A/G resin.

Table 2

Summarizing data on the yield of Nb5 and rPasB after purification with Nb5 or Bio-Nb5.

Purification strategy	Input of contaminated rPasB (μg)	Output of pure rPasB (μg)	Recovery percentage ^a (%)	Final yield of rPasB ^a (μg/g wet cells)
Strategy I: Nb5	50	16	45.7	10.7
Strategy II: Bio-Nb5	125	20	22.9	5.3

Percentage of SlyD in contaminated rPasB: ~30%
Yield of Nb5: 8.8 mg/L of culture medium
Yield of rPasB complex: 500 μg/L of culture medium

^a The percentage of recovery and final yield will depend also on the stability of the recombinant protein during the steps of purification. As rPasB is not stable at room temperature, a significant amount of rPasB will denature and precipitate over time.

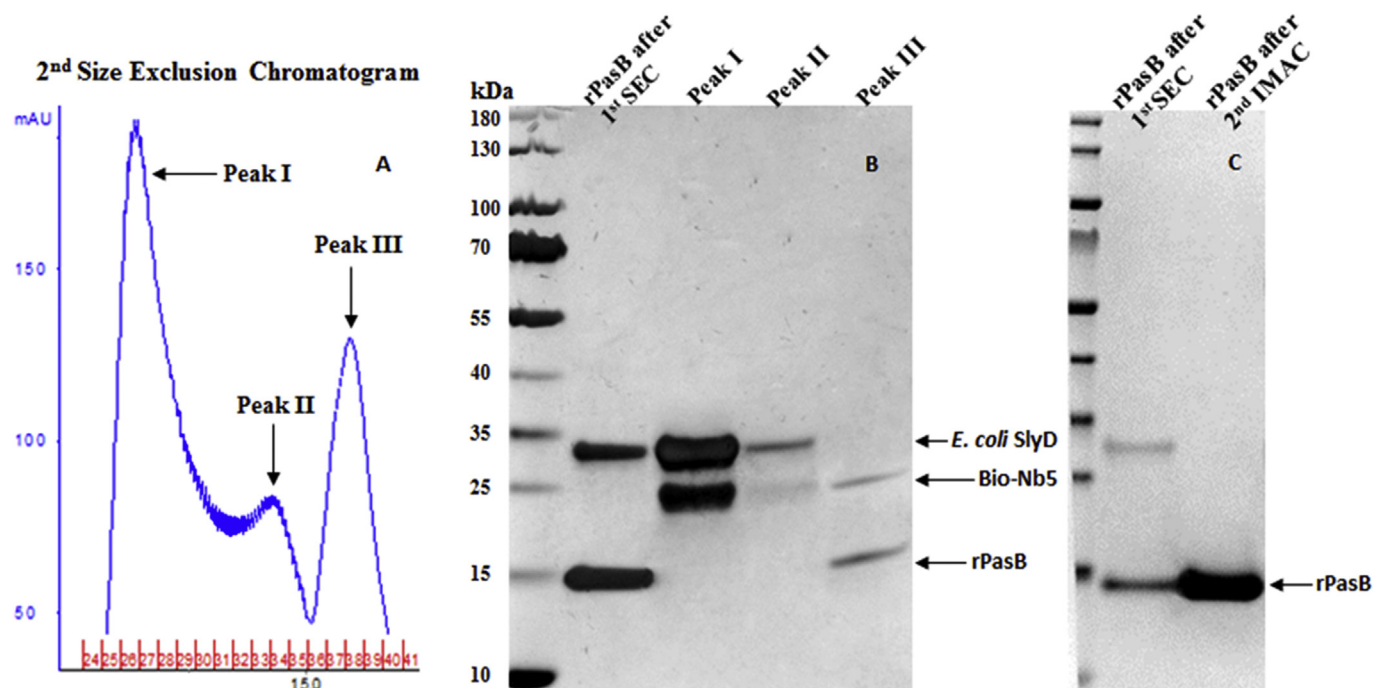


Fig. 7. Removal of contaminant SlyD from recombinant protein with Bio-Nb5 followed by SEC and IMAC purification. (A) The SEC profile of rPasB, SlyD and Bio-Nb5 mixture. Three fractions (denoted peak I, II and III) were taken to analyze their protein contents by SDS-PAGE, stained by Coomassie or western blot. (B) Proteins from peak fractions I, II and III stained with Coomassie. The first lane denoted rPasB after 1st SEC is the starting material before mixing with Bio-Nb5 and separation by 2nd SEC. Peak III is the only fraction that contains the rPasB, but it is still contaminated with Bio-Nb5. (C) Purity analysis of rPasB from Peak III after 2nd IMAC. The fraction corresponding to peak III containing rPasB and Bio-Nb5 was applied to a Ni-NTA column. The protein eluted with imidazole, was concentrated and analyzed by SDS-PAGE.

4. Discussion

The standard procedure to prepare recombinant proteins consists of designing the target gene, taking into account the preferred codon usage of the production host and tagging the gene with His codons to facilitate the purification of the expressed protein via IMAC. Unfortunately, some proteins are rather difficult to express in large quantities in *E. coli*, as is the case for our protein of interest, rPasB. Using standard IMAC and SEC purification procedures, the yield of rPasB remained low and consequently, the purified fraction still contained contaminating *E. coli* proteins. In our case we consistently observed a contaminating protein band with an apparent MW of around 28,000 on SDS-PAGE [19]. Attempts to include more stringent washing steps including solutions with 50 or even 100 mM imidazole on the Ni-NTA resin, or switching to purification on TALON, failed to eliminate selectively the contaminating protein as both, the contaminant protein and rPasB were systematically eluted simultaneously. As a result, we predicted that this contaminating protein would always remain present to some extent in any recombinant protein preparation (when recombinant protein has MW around 10–30 kDa) purified from an *E. coli* extract through IMAC and SEC.

Consequently, the immunization of a dromedary with such contaminated recombinant protein preparation has the risk that the animal will raise a variable immune response (in the heavy chain-only isotypes) to the target but also to the contaminants depending on the immunogenicity of each of them. Unfortunately, these bacterial contaminating proteins are evidently highly immunogenic. The VHH library constructed from the immunized camelid [24], will therefore contain a larger variety of binders against the contaminants rather than against the target, a situation that obviously needs to be avoided. In our case we retrieved after panning, multiple Nbs against the rPasB preparation, that could be grouped in 7 families. Members of only 2 Nb families were directed against our target rPasB and Nbs of 5 families recognized the contaminant. The Nbs against the contaminant were of good affinity with K_D -values in the low nM range. Nb5 showed the highest affinity for its target that was defined by LC-MS/MS to be the *E. coli* SlyD [36]. SlyD is a peptidyl prolyl *cis/trans*-isomerase (PPIase), a chaperone present in different *E. coli* strains. This SlyD protein contains 196 amino acids with a MW of 20,852 and a pI of 4.86. Remarkably, a stretch of 31 consecutive amino acids from position 149 till 179 contains 14 histidines (HGHVHGAHDHHHDHDHGDCCGGHGHHDHGHEH). This high

number of histidines in the C terminal region of SlyD, also known as the C-terminal metal binding domain, ensures the binding to Zn^{2+} and Ni^{2+} ions, as well as Cu^{2+} and Co^{2+} , *in vitro* [35] and clarifies its co-purification with His-tagged recombinant proteins on IMAC. SlyD is a natural chaperone, generally produced by *E. coli* expression hosts and the co-purification of this protein with recombinant proteins has been reported repeatedly [19,35]. It is therefore recognized as a potential permanent contaminant of purified His-tagged recombinant proteins, especially when the recombinant protein has a similar size to that of SlyD.

As a proof of principle, we developed two different strategies to remove *E. coli* SlyD from recombinant protein preparations. In the first approach, we utilize the SlyD-specific Nb5 with a C-terminal HA-tag. In the second protocol we employ the *in vivo* biotinylated Nb5. In both case, the Nb5 moiety serves to complex the SlyD protein from the recombinant protein mixture, where the HA- or biotin-tag is used to capture the complex. The HA-tagged immune complex is precipitated with Protein A/G beads decorated with the monoclonal anti-HA antibody [37]. The biotin-tagged immuno-complex is separated from the recombinant protein via SEC. In both cases, SDS-PAGE and western blot demonstrated the quantitative removal of SlyD.

The HA-tagged mediated strategy is time-saving, but restricted by the high cost of anti-HA monoclonal antibodies and Protein A/G beads. So, this approach is suitable to prepare rapidly, small quantities of pure recombinant protein. In contrast, the strategy to increase the MW of the SlyD fraction by forming an immune complex with Bio-Nb5 and its removal by SEC, is more time-consuming, but also more economic and is favored to prepare large batches of recombinant protein. Conversely, it will only work to purify recombinant proteins with MW < 20,000. In addition, although the recombinant protein is free from SlyD after SEC purification, it is now contaminated with residual free Bio-Nb5. By passing the mixture over IMAC, the Nb5 will be part of the flow-through fraction; whereas the Ni^{2+} bound recombinant protein can be eluted with imidazole, which then needs to be dialyzed out. Alternatively, the Bio-Nb5 material can also be removed via streptavidin-coated beads instead of IMAC, but this is again more expensive.

Obviously, several adaptations or combination on these SlyD elimination methods can be designed according to the downstream requirements. For example, one could reconstitute the Nb5 into a Heavy chain-only antibody to increase the size of the SlyD immune complex even further and thus eliminating the need of a subsequent IMAC; generating a bivalent Nb5 would also increase the MW of the complex and increase the functional affinity for SlyD as well. Finally, we propose to couple Nb5 covalently to a solid support (e.g., CNBr activated Sepharose or agarose, carboxyl-modified microspheres or latex beads) to produce an affinity adsorbent to purify large quantities.

We make our SlyD specific nanobodies available to the scientific research community upon justified request.

Conflict of interest

The authors declare to have no conflict of interests with the contents of this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pep.2017.06.016>.

References

- [1] G.L. Rosano, E.A. Ceccarelli, Recombinant protein expression in *Escherichia coli*: advances and challenges, *Front. Microbiol.* 5 (2014) 172.
- [2] J.L. Adrio, A.L. Demain, Recombinant organisms for production of industrial products, *Bioeng. Bugs* 1 (2010) 116–131.
- [3] R.C. Cheung, J.H. Wong, T.B. Ng, Immobilized metal ion affinity chromatography: a review on its applications, *Appl. Microbiol. Biotechnol.* 96 (2012) 1411–1420.
- [4] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Metal chelate affinity chromatography, a new approach to protein fractionation, *Nature* 258 (1975) 598–599.
- [5] E. Hochuli, H. Dobeli, A. Schacher, New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues, *J. Chromatogr.* 411 (1987) 177–184.
- [6] M.C. Smith, T.C. Furman, T.D. Ingolia, C. Pidgeon, Chelating peptide-immobilized metal ion affinity chromatography. A new concept in affinity chromatography for recombinant proteins, *J. Biol. Chem.* 263 (1988) 7211–7215.
- [7] A.K. Mohanty, M.C. Wiener, Membrane protein expression and production: effects of polyhistidine tag length and position, *Protein Expr. Purif.* 33 (2004) 311–325.
- [8] M.B. Murphy, S.A. Doyle, High-throughput purification of hexahistidine-tagged proteins expressed in *E. coli*, *Methods Mol. Biol.* 310 (2005) 123–130.
- [9] X. Sun, J.F. Chiu, Q.Y. He, Application of immobilized metal affinity chromatography in proteomics, *Expert Rev. Proteomics* 2 (2005) 649–657.
- [10] J. Crowe, H. Dobeli, R. Gentz, E. Hochuli, D. Stuber, K. Henco, 6xHis-ni-nta chromatography as a superior technique in recombinant protein expression/purification, *Methods Mol. Biol.* 31 (1994) 371–387.
- [11] H. Li, X. Gao, Y. Zhou, N. Li, C. Ge, X. Hui, Y. Wang, A. Xu, S. Jin, D. Wu, High level expression, purification and characterization of active fusion human c1q and tumor necrosis factor related protein 2 (hctrp2) in *Escherichia coli*, *Protein Expr. Purif.* 79 (2011) 1–6.
- [12] V. Gaberc-Porekar, V. Menart, Perspectives of immobilized-metal affinity chromatography, *J. Biochem. Biophys. Methods* 49 (2001) 335–360.
- [13] C. Mateo, G. Fernandez-Lorente, B.C. Pessela, A. Vian, A.V. Carrascosa, J.L. Garcia, R. Fernandez-Lafuente, J.M. Guisan, Affinity chromatography of polyhistidine tagged enzymes. New dextran-coated immobilized metal ion affinity chromatography matrices for prevention of undesired multipoint adsorptions, *J. Chromatogr. A* 915 (2001) 97–106.
- [14] Y. Cai, M. Moore, R. Goforth, R. Henry, R. Beitle, Genomic data for alternate production strategies. I. Identification of major contaminating species for cobalt(+2) immobilized metal affinity chromatography, *Biotechnol. Bioeng.* 88 (2004) 77–83.
- [15] C.T. Wehr, R.L. Cunico, G.S. Ott, V.G. Shore, Preparative size-exclusion chromatography of human serum apolipoproteins using an analytical liquid chromatograph, *Anal. Biochem.* 125 (1982) 386–394.
- [16] C. Scholz, B. Eckert, F. Hagn, P. Schaarschmidt, J. Balbach, F.X. Schmid, SlyD proteins from different species exhibit high prolyl isomerase and chaperone activities, *Biochemistry* 45 (2006) 20–33.
- [17] M. Kovermann, F.X. Schmid, J. Balbach, Molecular function of the prolyl cis/trans isomerase and metallochaperone slyd, *Biol. Chem.* 394 (2013) 965–975.
- [18] T. Mitterauer, C. Nanoff, H. Ahorn, M. Freissmuth, M. Hohenegger, Metal-dependent nucleotide binding to the *Escherichia coli* rotamase slyd, *Biochem. J.* 342 (Pt 1) (1999) 33–39.
- [19] V.M. Bolanos-Garcia, O.R. Davies, Structural analysis and classification of native proteins from *E. coli* commonly co-purified by immobilised metal affinity chromatography, *Biochim. Biophys. Acta* 1760 (2006) 1304–1313.
- [20] S. Hottenrott, T. Schumann, A. Pluckthun, G. Fischer, J.U. Rahfeld, The *Escherichia coli* slyd is a metal ion-regulated peptidyl-prolyl cis/trans-isomerase, *J. Biol. Chem.* 272 (1997) 15697–15701.
- [21] K.L. Franken, H.S. Hiemstra, K.E. van Meijgaarden, Y. Subronto, J. den Hartigh, T.H. Ottenhoff, J.W. Drijfhout, Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent, *Protein Expr. Purif.* 18 (2000) 95–99.
- [22] C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E.B. Songa, N. Bendahman, R. Hamers, Naturally occurring antibodies devoid of light chains, *Nature* 363 (1993) 446–448.
- [23] G. Hassanzadeh-Ghassabeh, N. Devoogdt, P. De Pauw, C. Vincke, S. Muyldermans, Nanobodies and their potential applications, *Nanomedicine Lond. Engl.* 8 (2013) 1013–1026.
- [24] S. Muyldermans, Nanobodies: natural single-domain antibodies, *Annu. Rev. Biochem.* 82 (2013) 775–797.
- [25] J. Helma, M.C. Cardoso, S. Muyldermans, H. Leonhardt, Nanobodies and recombinant binders in cell biology, *J. Cell Biol.* 209 (2015) 633–644.
- [26] O.Y. Dmitriev, S. Lutsenko, S. Muyldermans, Nanobodies as probes for protein dynamics *in vitro* and in cells, *J. Biol. Chem.* 291 (2016) 3767–3775.
- [27] E. Romao, F. Morales-Yanez, Y. Hu, M. Crauwels, P. De Pauw, G.G. Hassanzadeh, N. Devoogdt, C. Ackaert, C. Vincke, S. Muyldermans,

- Identification of useful nanobodies by phage display of immune single domain libraries derived from camelid heavy chain antibodies, *Curr. Pharm. Des.* 22 (2016) 6500–6518.
- [28] C. Vincke, S. Muyldermans, Introduction to heavy chain antibodies and derived nanobodies, *Methods Mol. Biol.* 911 (2012) 15–26.
- [29] N. Gomez-Roman, N.M. Sahasrabudhe, F. McGregor, A.J. Chalmers, J. Cassidy, J. Plumb, Hypoxia-inducible factor 1 alpha is required for the tumourigenic and aggressive phenotype associated with rab25 expression in ovarian cancer, *Oncotarget* 7 (2016) 22650–22664.
- [30] G.N. Masoud, W. Li, Hif-1alpha pathway: role, regulation and intervention for cancer therapy, *Acta Pharm. Sin. B* 5 (2015) 378–389.
- [31] S. Adinolfi, C. Bagni, G. Musco, T. Gibson, L. Mazzarella, A. Pastore, Dissecting *fmr1*, the protein responsible for fragile x syndrome, in its structural and functional domains, *RNA* 5 (1999) 1248–1258.
- [32] C.M. Lee, N. Iorno, F. Sierro, D. Christ, Selection of human antibody fragments by phage display, *Nat. Protoc.* 2 (2007) 3001–3008.
- [33] A. Skerra, A. Pluckthun, Assembly of a functional immunoglobulin fv fragment in *Escherichia coli*, *Science* 240 (1988) 1038–1041.
- [34] H. Zhao, D. Beckett, Kinetic partitioning between alternative protein-protein interactions controls a transcriptional switch, *J. Mol. Biol.* 380 (2008) 223–236.
- [35] C. Wulfig, J. Lombardero, A. Pluckthun, An *Escherichia coli* protein consisting of a domain homologous to fk506-binding proteins (fkbp) and a new metal binding motif, *J. Biol. Chem.* 269 (1994) 2895–2901.
- [36] L. Chang, J. Ni, J. Beretov, V.C. Wasinger, J. Hao, J. Bucci, D. Malouf, D. Gillatt, P.H. Graham, Y. Li, Identification of protein biomarkers and signaling pathways associated with prostate cancer radioresistance using label-free lc-ms/ms proteomic approach, *Sci. Rep.* 7 (2017) 41834.
- [37] A.J. Groot, P. Verheesen, E.J. Westerlaken, E.H. Gort, P. van der Groep, N. Bovenschen, E. van der Wall, P.J. van Diest, A. Shvarts, Identification by phage display of single-domain antibody fragments specific for the odd domain in hypoxia-inducible factor 1alpha, *Lab. Invest.* 86 (2006) 345–356.